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Liquid Interferon-β Formulations

Description

The present invention relates to liquid formulations of human interferon-β. These formulations are characterized in that they have a pH value between 5 and 8 in the weakly acidic to neutral range, and they have a high stability of the interferon-β in solution while retaining its molecular integrity.

Naturally occurring interferons are species-specific proteins, in some cases glycoproteins, which are synthesized by various cells in the body after induction with viruses, double-stranded RNA, other polynucleotides and antigens. Interferons have numerous biological activities such as antiviral, antiproliferative and immunomodulatory properties. At least three different types of human interferons have been identified; these interferons, which are produced by leukocytes, lymphocytes, fibroblasts and cells of the immune system, are referred to as α-, β-, and γε-interferons. Individual types of interferons may also be subdivided into various subtypes.

Native human interferon-β can be produced industrially by superinduction of human fibroblast cell cultures with poly-IC and subsequent isolation and purification of interferon β by chromatographic and electrophoretic techniques. Proteins or polypeptides having properties comparable to those of natural interferon-β can also be produced by recombinant DNA technologies (European Patent Application EP-A-0 028 033; European Patent Application EP-A-0 041 313; European Patent Application EP-A-0 070 906; European Patent Application EP-A-0 287 075; Chernajovsky et al. (1984), *DNA*, 3, 297-308; McCormick et al. (1984), *Mol. Cell. Biol.*, 4, 166-172). Recombinant human interferon-β can be produced in both eukaryotic cells (e.g., CHO cells) and prokaryotic cells (e.g., *E. coli*). The corresponding interferons are known as interferon-β-1a and interferon-β-1b. In contrast with interferon-β-1b, interferon-β-1a is glycosylated (Goodkin (1994), *Lancet*, 344, 1057-1060).

Therapeutic use of interferon-β presupposes that it is converted to a pharmaceutical preparation which permits storage of the protein for a long period of time while maintaining its molecular integrity. Interferon-β is unstable and is subject to various degradation reactions, including in particular, cleavage of peptide bonds, deamidation, oxidation of methionine to methionine sulfide, disulfide exchange and changes in the sugar side chains up to and including deglycosylation.

On the basis of the therapeutic benefit of interferons, a number of formulations have been developed in past years, but they all had certain disadvantages. U.S. Patent No. 4,647,454 (Inter Yeda Ltd.) describes a formulation of fibroblast interferon-β which can be stabilized by adding polyvinyl pyrrolidone (PVP) in the strongly acidic range (pH 3.5). Other preferred additives include mannitol, human serum albumin and acetate buffer. The formulation is freeze-dried and stored at 4 °C.

Japanese Patent 59 181 224 (Sumitomo Chemical Co.) describes an aqueous solution of interferons in which polar amino acids such as arginine, asparagine, glutamic acid, glutamine, histidine, lysine, serine and threonine or their sodium salts are used together with human serum albumin to stabilize the interferons.

International Patent Application WO 95/31213 (Applied Research Systems ARS Holding) describes a liquid formulation for interferon-β, which is stabilized by adding a polyol, preferably mannitol, and a non-reducing sugar or an amino acid. The formulation also contains a buffer (acetate buffer, pH 3.0 to 4.0) as well as human serum albumin. Although formulations having a pH value between 5 and 6 have shown an immediate loss of biological activity, the formulations preferred in this patent publication have sufficient stability at a pH value of 3.0 or 4.0. In this case, the stability is evaluated on the basis of the biological activity of the formulation but not the molecular integrity of the active ingredient.

European Patent Application EP 0 215 658 (Cetus Corp.) describes a formulation for recombinant interferon-β in which the biologically active compound is dissolved in an aqueous medium at a pH between 2 and 4 with the addition of stabilizers such as human serum albumin or human plasma protein fractions and optionally dextrose. Another patent application by Cetus Corp. (International Patent WO 89/05158) describes an interferon-β formulation which has a pH between 2 and 4 and uses as stabilizers either glycerol or polyethylene glycol polymers with an average molecular weight between 190 and 1600 daltons. Suitable buffer components include glycine, phosphoric acid and citric acid.

European Patent EP 0 217 645 (Cetus Corp.) describes pharmaceutical preparations containing IL-2 or interferon-β which are dissolved in a carrier medium at a pH of 7 to 8 and are stabilized with the addition of sodium laurate as the surface-active compound. In addition, SDS is also needed as an additional ionic surfactant compound to stabilize these preparations.

European Patent EP 0 270 799 (Cetus Oncology Corp.) describes a formulation for nonglycosylated recombinant interferon-β in an inert carrier medium based on water, containing nonionic polymer detergents as stabilizers.

European Patent Application EP 0 529 300 (Rentschler Biotechnologie GmbH) describes liquid interferon-β formulations which contain recombinant IFN-β in a concentration of 30 or 70 MU/mL plus sodium chloride and imidazole or sodium phosphate buffer at a pH of 7.5 (Example 3). These formulations are stable for four weeks at a storage temperature of 25 °C with respect to their biological activity. However, these combinations have the disadvantage that the concentration of interferon-β used in them ($\geq$ 30 MU/mL) is too high for practical applications. In addition, there is no mention in European Patent Application EP-A 0 529 300 that the stability of liquid interferon-β formulations is reduced by the addition of human serum albumin. On the contrary, the addition of human serum albumin is mentioned as preferred.

In addition to formulations of interferon-β, pharmaceutical forms of administration containing interferon-ϒ have also been described. European Patent 0 082 481 (Schering Corp.) discloses an aqueous formulation intended for freeze drying which contains human serum albumin in addition to a phosphate buffer. Alanine is mentioned as another optional ingredient. The pH value of the solution after reconstitution is between 7.0 and 7.4. Another patent application by Schering Corp. (International Patent WO 96/11018) discloses stable aqueous solutions of interferon-α which contain (at a pH between 4.5 and 7.1) chelating agents (NaEDTA or citric acid), a surfactant compound (polysorbate 80), an isotonizing agent (sodium chloride) as well as suitable preservatives such as methylparaben, propylparaben, *m*-cresol or phenol. The aqueous formulations disclosed there have proven to be stable (biological activity > 90% of the starting activity) with regard to their biological activity for six months at 25 °C (standard method of inhibition of the cytopathic effect (CPE) of a virus as described by W. P. Protzman in *J. Clinical Microbiology*, 1985, 22, pp. 596-599). However, a parallel determination of the protein content by HPLC shows a decline in content amounting to between 20.2 % (Table 3) and 32.5 % (Table 4) after only six months at 25 °C.

European Patent Application EP-A 0 736 303 (Hoffmann LaRoche AG) discloses aqueous interferon-ϒ compositions which contain, in addition to an interferon-α, a nonionic detergent, a buffer for adjusting the pH value to a range between 4.5 and 5.5, benzyl alcohol and an optional isotonizing agent. With an initial concentration of 18 MU interferon-αZ2e, a residual interferon content of 84.5 % is found in a determination by means of HPLC after three months of storage at 25 °C, but this value drops to 62.8 % when benzyl alcohol is omitted as the stabilizer.

European Patent Application EP-A 0 641 567 (Ciba Geigy AG) describes pharmaceutical compositions containing hybrid interferon-α and, as the stabilizer, a buffer at a pH between 3.0 and 5.0.

U.S. Patent 5,358,708 (Schering Corp.) describes aqueous formulations of interferon-α containing as the stabilizer methionine, histidine or mixtures thereof. After two weeks of storage of an interferon-α solution at 40 °C, a 20 % drop in the active ingredient content is found.

According to the prevailing standpoint today, the formulations of interferons listed above have numerous disadvantages, because the addition of human serum albumin should now be avoided for stabilization of proteins because of increased demands regarding safety from viral contamination due to blood donors. In addition, for a number of the formulations described above, it is also necessary to add amino acids and/or to perform freeze-drying. However, freeze-dried products are very complicated to manufacture and are expensive accordingly, and they require an additional step due to the need for reconstitution; however, this is often difficult to do for patients with restricted motor capacity. A number of formulations have a non-physiological pH value of less than 5.0. Although such values are not completely unconventional (see also S. Sweetans and N. J. Aders, *Journal of Pharmaceutical Sciences and Technology*, 1996, 50:330-342), painful irritation must be expected when these preparations are administered intramuscularly or subcutaneously. Although it is admissible to use surfactant compounds such as polysorbate 80 (according to Sweetana and Akers), however, a number of side effects have been described, especially in children

and neonates, casting doubt on the use of such additives. The toxicity of surfactant compounds is reported in a summary form by Attwood and Florence (*Surfactant Systems, Their Chemistry, Pharmacy and Biology,* Chapman and Hall; London, 1983). A review of the pharmacology of polysorbate 80 can be found in R. K. Varma et al. (*Arzneim.-Forsch./Drug Research*, 35, 1985, 804-808).

Because of the disadvantages mentioned above, an optimum formulation for interferon-β should combine the following properties:

-Preserving the biological activity over the entire storage period,

-Preserving the molecular integrity of the active ingredient molecule over the entire storage period,

-Liquid formulation, no expensive freeze drying or additional reconstitution,

-Avoiding high-risk additives such as human serum albumin or surfactant compounds (detergents),

-pH value in the neutral to weakly acidic range.

All requirements are met by the present invention, which is described in greater detail in the following section.

Surprising, a formulation has been discovered which ensures the molecular integrity of interferon-β in liquid form over a long period of time in a physiological pH range between 5 and 8, preferably from 5.5 to 8 without having to rely on the known additives of the state of the art.

Another aspect of the present invention is therefore a liquid pharmaceutical formulation which contains human interferon-β as an active ingredient in a concentration of up to 25 MU/mL and a buffer to establish a certain pH value, preferably between greater than 5.5 and 8, is free of human serum albumin, and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

Another aspect of this invention is a liquid pharmaceutical formulation which contains human interferon-β as the active ingredient and a buffer for adjusting the pH to a value between 6 and 7.2, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

Yet another aspect of this invention is a liquid pharmaceutical formulation which contains human IFN- β as the active ingredient, a buffer for adjusting a pH between 5 and 8, preferably between greater than 5.5 and 8, contains one or more amino acids and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months at 25 °C.

The long-term stability of liquid pharmaceutical formulations was measured at 25 °C. A temperature of 25 °C was selected to accelerate degradation reactions on the one hand while on the other hand not causing any artefacts to be formed due to excessive temperatures. Suitable analytical methods for determining the stability of interferon data are described in the review article by J. Geigert (*J. Parent. Sci. Technol.* 43 (1989) 220-224) or M. C. Manning, K. Patel and R. T. Borchardt (*Pharm. Res.* 6 (1989), 903-918).

The biological activity was measured after the specified storage period by the standard method of inhibition of the cytopathic effect of a virus. A precise description of the test method used can be found in W. E. Stewart II (1981): *The Interferon System* (second enlarged edition), Springer Verlag, Vienna, New York; S. E. Grossberg et al. (1984), "Assay of Interferons," in: P. E. Came, W. A. Carter (eds.), *Interferons and Their Applications*, Springer Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 23-43.
After three months of storage at 25 °C, a formulation according to this invention will have a biological activity of at least 80 %, preferably at least 85 %, and especially preferably at least 90 % of the initial activity.
A formulation according to this invention will preferably have a biological activity of at least 80 % and preferably at least 85 % of the initial activity after storage for six months at 25 °C.
Even in the case of storage at a higher temperature, such as 37 °C, the formulations according to this invention have a surprisingly high long-term stability of the biological activity. Thus, after storage for one month at 37 °C, a biological activity of at least 70 % and preferably at least 80 % of the initial activity was found.
The liquid pharmaceutical formulations according to this invention are preferably free of human serum albumin and especially preferably free of human or animal polypeptides (apart from the active ingredient), especially serum proteins. In addition, it is preferable for the liquid pharmaceutical formulation according to this invention to be free of surfactant agents, and in particular free of ionic detergents and/or nonionic surfactants.
The formulations according to this invention contain as the active ingredient an interferon-β , i.e., a polypeptide which has the biological and/or immunological properties of natural human interferon-β and may be a naturally occurring or recombinant interferon-β . The formulation preferably contains a glycosylated interferon-β , especially preferably a recombinant interferon-β from CHO cells. The most preferred are interferon-β species such as those obtained from the cell line BiC 8622 (ECACC 87 04 03 01) and described in European Patent EP-B-0 287 075 and European Patent Application EP-A-0 529 300, for example.
The active ingredient is preferably present in the formulations according to this invention in a concentration of up to 25 MU/mL. However, a dosage in the range of 1 to 25 MU/mL is preferred, especially preferably from 3 to 20 MU/mL and most preferably from 3 to 10 MU/mL. These dosage ranges allow direct use without further dilution plus an especially good stability at elevated temperatures.
Another preferred feature of the liquid pharmaceutical formulation according to this invention is that it has chemical integrity after storage at 25 °C for three months and preferably for six months, i.e., it is stable with respect to peptide cleavage, oxidation and deglycosylation. The chemical integrity is measured by peptide mapping, Western Blot and glycosylation analysis. Compositions in which the interferon-β retains at least 85 %, preferably at least 90 % of its chemical integrity under the selected storage conditions are considered to be chemically stable in conjunction with the present invention.
Another preferred feature of the liquid pharmaceutical formulations according to this invention is their physical integrity after storage for three months at 25 °C and preferably for six months. The physical

integrity is measured by measuring the transmittance at 420 nm and by visual observation of the solutions. The solutions which are physically stable are those whose transmittance is more than 90 %, preferably more than 93 % under the selected storage conditions and in which no turbidity can be detected by visual observation.

Surprisingly, liquid formulations of interferon-β which are biologically, chemically and physically stable over a long period of time and are free of unwanted ingredients such as human serum albumin or surfactant agents can be made available through the present invention. The formulations according to this invention contain, in addition to the active ingredient, a buffer which is preferably present in a concentration of 10 mmol/L to 1 mol/L, especially preferably in a concentration of 20 mmol/L to 200 mmol/L, e.g., approx. 50 mmol/L to 100 mmol/L, and which serves to keep the pH of the formulation in the range of 5 to 8, preferably from more than 5.5 to 8 and even more preferably between 6 and 7.4. A pH between 6 and 7.2 is especially preferred, and a pH between 6.2 and 6.8 is most preferred, because this is where an especially high stability is achieved while maintaining molecular integrity. The buffer is selected from pharmaceutically acceptable buffers such as borate, succinate, L-malate, Tris, salicylate, glycylglycine, triethanolamine, isocitrate, maleate, phosphate, citrate and acetate buffers or mixtures thereof. The preferred buffers are phosphate, citrate and acetate buffers or mixtures thereof, especially preferably phosphate/citrate buffer.

In addition to the active ingredient and the buffer, the formulation according to this invention may also contain other physiologically safe additives such as additives to adapt the tonicity to that of blood or tissue, such as non-reducing sugars, sugar alcohols such as mannitol, sorbitol, xylitol or glycerol. In addition, one or more amino acids such as alanine, arginine, glycine, histidine and/or methionine may be added to the formulation according to this invention to further increase its chemical stability. Methionine is preferred here. The methionine concentration is preferably in the range of 0.1 to 4 mmol/L. A concentration of 2 mmol/L is especially preferred. In addition, the formulation may contain thickeners to increase the viscosity, e.g., for ophthalmological applications. Examples of suitable thickeners include ophthalmologically suitable polymers such as carbopol, methylcellulose, carboxymethylcellulose, etc.

In addition, the composition according to this invention may also contain preservatives. For ophthalmological purposes, thiomersal, for example, may be used in an amount of 0.001 % to 0.004 % (weight/volume).

In addition, this invention also relates to pharmaceutical preparations which contain a liquid formulation of interferon-β as described above. These pharmaceutical preparations are especially suitable for oral, parenteral or ophthalmological application. These formulations are preferably prepared in single doses of 1 to 25 MU IFN- β. In addition, this invention also relates to a method of producing such pharmaceutical preparations, wherein a formulation according to this invention plus optionally other necessary pharmaceutically additives is prepared and converted to a suitable form of administration.

The formulation according to this invention can be stored in suitable washed and sterilized glass vials (hydrolytic class 1) with pharmaceutically acceptable rubber stoppers.

In addition, formulations according to this invention may also be packaged aseptically in ready-to-use syringes or in cartridge ampoules for use in self-injection systems. The aqueous solutions may be freeze

dried (although this is not preferred) by adding other additives with which those skilled in the art are familiar, and are then available in liquid form after being reconstituted.
Liquid multiple dosage forms and eyedrop solutions and drop solutions for oral administration may be prepared by adding suitable preservatives.
The additional additives needed for production of the corresponding forms of administration are familiar to those skilled in the art.
Finally, this invention relates to a method of improving the stability of the liquid formulation which contains human interferon-β as the active ingredient and a buffer for adjusting the pH value in the range of 5 to 8, preferably from greater than 5.5 to 8, characterized in that a formulation without human serum albumin and/or with one or more amino acids is used. The improvement in stability includes an improvement in the long-term stability of the biological activity (*in vitro*), the chemical integrity and/or the physical integrity as described above.
In addition, this invention is illustrated by the following examples.

Examples

An interferon-β obtained from CHO cells was used in all the examples.

1. Long-term stability of liquid interferon-β formulations at 25 °C

The following formulations were tested:

Formulation 1: 50 mmol/L sodium citrate, pH 5.0

Formulation 2: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 15 mg/mL human serum albumin, 2 mmol/L methionine, 50 mg/mL glycerol

Formulation 3: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 50 mg/mL glycerol, 2 mmol methionine

Formulation 4: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 2 mmol/L methionine

Formulation 5: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0

Formulation 17: 70 mmol/L sodium citrate, 50 mmol/L sodium phosphate, 2 mmol/L methionine, pH 6.5

The formulations were diluted to a content of approx. 10 to 15 MU/mL (i.e., 10 to 15 × 10^6 IU/mL). With the exception of formula 17 (see below), the formulations were placed in glass vials of hydrolytic class 1 (DIN 2R vials) which were sealed with conventional chlorobutyl rubber stoppers and stored at 25 °C for the stated period of time. The biological activity (*in vitro*) was determined by the method described by W. E. Stewart II (1981): *The Interferon System* (second enlarged edition), Springer Verlag: Vienna New York; S. E. Grossberg et al. (1984), "Assay of Interferons," in: P. E. Came, W. A. Carter (eds.), *Interferons and their Applications*, Springer Verlag: Berlin, Heidelberg, New York, Tokyo, pp. 23-43.

The results are shown in Tables 1 through 5. The notation "% (Ref)" refers to the relative biological activity based on the biological activity of a reference specimen for the stated period of time at -20 °C. The notation "% (0Mo)" refers to the percentage biological activity based on the initial value at 0 months.

Table 1 (Formulation 1):

Months	Active ingredient content			
			Recovery (25 °C)	
	-20 °C	25 °C	% (Ref)	% (0 Mo)
0	11.0	11.0	100	100
1	10.0	9.8	98	89
2	9.7	11.0	113	100
3	10.0	10.6	106	96
4	10.3	9.5	92	86
5	9.5	9.7	102	88
6	10.5	10.2	97	93

Table 2 (Formulation 2):

Months	Active ingredient content			
			Recovery (25°C)	
	-20 °C	25 °C	% (Ref)	% (0 Mo)
0	13.9	13.9	100	100
1	14.0	11.9	85	86
2	13.0	11.6	89	83
3	13.1	9.6	73	69
4	12.5	8.8	70	63
5	11.0	8.2	75	59
6	13.3	8.4	63	60

Table 3 (Formulation 3):

Months	Active ingredient content			
			Recovery (25 °C)	
	-20 °C	25 °C	% (Ref)	% (0 Mo)
0	12.5	12.5	100	100
1	9.4	10.0	106	80
2	8.3	11.5	139	92
3	7.8	11.8	151	94.4
4	6.8	10.3	151	82.4
5	6.6	11.2	170	89.6
6	7.8	13.4	172	107.2

Table 4 (Formulation 4):

Months	Active ingredient content			
			Recovery (25 °C)	
	-20 °C	25 °C	% (Ref)	% (0 Mo)
0	11.4	11.4	100	100
1	10.5	10.2	97	89
2	11.9	11.1	93	97
3	10.8	10.0	93	88
4	10.4	9.3	89	82
5	11.6	8.4	72	74
6	12.4	9.5	77	83

Table 5 (Formulation 5):

Months	Active ingredient content			
			Recovery (25 °C)	
	-20 °C	25 °C	% (Ref)	% (0 Mo)
0	11.3	11.3	100	100
1	11.0	9.7	88	86
2	11.7	10.1	86	89
3	11.1	10.2	92	90
4	11.3	10.2	90	90
5	12.0	9.2	77	81
6	11.0	9.7	88	66

It can be seen from the above tables that formulations which do not contain any human serum albumin (formulations 1, 3, 4 and 5) surprisingly have a better stability than a formulation (formulation 2) which contains human serum albumin.

In the case of formulation 17 (see above), an interferon solution without human serum albumin was adjusted to an activity of 6 MU/0.5 mL under aseptic conditions. The clear, colorless solution was then filtered under sterile conditions and packaged in 0.5 mL amounts in pre-sterilized disposable syringes and sealed. The ready-to-use syringes were stored at 25 °C and were tested for clarity, pH and biological activity, yielding the following results:

Storage in months	pH	Clarity (%)	MU/syringe		Recovery (25 °C)	
			-20 °C	25 °C	% (Ref)	% (0 Mo)
0	6.5	99.5	6.3	6.3	100	100
3	6.5	99.1	5.6	6.1	108	97

2. Long term stability of liquid IFN-β formulations at 37 °C

The following formulations were tested in ready-to-use syringes:

Formulation 6: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0, 2 mmol/L methionine

Formulation 7: 50 mmol/L sodium citrate, pH 5.0, 18 mg/mL glycerol, 2 mmol/L methionine

Formulation 8: 50 mmol/L sodium citrate, pH 5.0, 18 mg/mL glycerol, 15 mg/mL human serum albumin, 2 mmol/L methionine

Formulation 9: 50 mmol/L sodium citrate, pH 6.0, 18 mg/mL glycerol, 2 mmol/L methionine

Formulation 10: 50 mmol/L sodium citrate, pH 6.5, 18 mg/mL glycerol, 2 mmol/L methionine

The formulations were tested in dosages of 3 MU per 0.5 mL (dosage strength 3), 6 MU per 0.5 mL (dosage strength 6) and 12 MU per mL (dosage strength 12).

The results are shown in the following Table 6.

Table 6.

Storage in months	Dosage strength 3					Dosage strength 6					Dosage strength 12				
	Formulation					Formulation					Formulation				
	6	7	8	9	10	6	7	8	9	10	6	7	8	9	10
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1	71	80	61	74	69	72	85	63	86	84	87	88	71	76	84
2	51	82	33	74	85	51	81	43	80	76	69	68	48	77	81
3	44	76	23	63	65	48	64	36	73	69	66	72	35	80	81
4	33	51	16	51	61	46	65	26	84	--	--	64	24	78	79

The results in Table 6 show that the formulations according to this invention without human serum albumin surprisingly have an improved stability at 37 °C.

3. Chemical stability at 25 °C

To investigate the chemical stability of liquid formulations of IFN-β , seven batches were formulated and stored at 25 °C. After storage for three months and six months, the protein was characterized by means of Lys-C mapping and a complete carbohydrate analysis. Special attention was devoted to the formation of methionine sulfoxide and to desialylation.

In addition to formulation 10 (see above), the following formulations were tested:

Formulation 11: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, 2 mmol/L methionine, pH 7.0 to 7.2

Formulation 12: 50 mmol/L sodium citrate, 50 mmol/L sodium phosphate, pH 7.0 to 7.2

Formulation 13: 50 mmol/L sodium citrate, 18 mg/mL glycerol, 2 mmol/L methionine, pH 5.0 to 5.2

Formulation 14: 50 mmol/L sodium citrate, 18 mg/mL glycerol, pH 5.0 to 5.2

Formulation 15: 50 mmol/L sodium citrate, 15 mg/mL human serum albumin (medical grade), 18 mg/mL glycerol, 2 mmol/L methionine, pH 5.0 to 5.2

Formulation 16: 50 mmol/L sodium citrate, 15 mg/mL human serum albumin (medical grade), 18 mg/mL glycerol, pH 5.0 to 5.2 (comparison)

In all batches, the IFN-β content was between 10 and 11 MU/mL.

Test procedure

To perform the analysis, it was necessary to increase the concentration of the specimens. In addition, in the case of batches 15 and 16, the human serum albumin had to be removed. Therefore, the batches were applied to an anti-β chromatography column. The starting volume per batch was 32 mL. Batches 13 to 16 were neutralized by adding 2.1 mL 0.4 mol/L Na_2HPO_4 and 2.1 mL 0.4 mol/L Na_2PO_4 before performing anti-β chromatography.

For the immunoadsorption of interferon-β on a monoclonal antibody to interferon-β (BO2 Sepharose 6B, crosslinked, from Celltech), a C10 chromatography column (Pharmacia) was packed with 5 mL BO2 Sepharose and rinsed three times with, each time, 5-10 gel volumes of PBS, 0.1 mol/L sodium phosphate, pH 2.0 and PBS/1 mol/L KCl at a linear flow rate of 1.0 cm/min.

Approx. 32 mL of the solution containing interferon/HSA was applied at a linear flow rate of 0.5 cm/min. The column was washed with 10 gel volumes of PBS/1 mol/L KCl at a linear flow rate of 1 cm/min until the OD dropped to the base line. Elution was performed with approx. 1-2 gel volumes of 0.1 mol/L sodium phosphate, pH 2, at a linear flow rate of 1 cm/min. Interferon-β was obtained as a single peak in a high purity. This eluate was suitable for the subsequent protein characterization.

Performing the analysis

1. Lys-C mapping

With the endoproteinase enzyme Lys-C from Achromobacter (AP), interferon-β was split into 12 peptides at the C-terminal end of lysine under reducing conditions.

Fifty µL eluate from anti-β chromatography (12.5-50 ϒg interferon-β) was placed in an Eppendorf reaction vessel and mixed with 5 µL 2 mol/L Tris. To this was added endoproteinase from the Wako company in an enzyme/substrate ratio of 1:10 (endoproteinase-Lys-C solution in 50 mmol/L Tris/HCl, pH 9.0). The solution was mixed and incubated for two hours at 30 °C. Then 5 µL of 0.1 mol/L DTT was added to the batch.

The peptides were separated on a reversed phase column (Vydac C18, 300 Å, 5 µm, 2.1 mm) using an HP 1090 M series HPLC system with a diode array detector at 214 mm [sic; nm], using a gradient of A: 0.1% (v/v) TFA and B: 0.1 (v/v) TFA/70% (v/v) acetonitrile. The peptides were numbered consecutively in the order of their retention times and were assigned to the following sequences.

Sequence ID No.	Peptide	Position	Sequence
1	AP1	109-115	EDFTRGK
2	AP2	100-105	TVLEEK
3	AP3	46-52	QLQQFQK
4	AP4(ox)	116-123	LM(ox)SSLHLK
5	AP4	116-123	LMSSLHLK
6	AP6(ox)	34-45	DRM(ox)NFDIPEEIK
7	AP5	124-134	RYYGRILHYLK
8	AP6	34-45	DRMNFDIPEEIK
9	AP7	20-33	LLWQLNGRLEYCLK
10	AP8(ox)	1-19	M(ox)SYNLLGFLQRSSNFQCQK
11	AP8	1-19	MSYNLLGFLORSSNFODQK
12	AP9	137-166	EYSHCAWTIVRVEILRNFYRNRLTGYLAN
13	AP10(ox)	53-99	EDAALTIYEM(ox)LQNIFAIFRQDSSS

			TGWENETIVENLLANVYHQINHLK
14	AP10	53-99	EDAALTIYEMLQNIFAIFRQDSSS TGWNETIVENLLANVYHQINHLK

Literature:

Utsumi et al. (1989). Characterization of four different mammalian-cell-derived recombinant human interferon-β1. *Eur. J. Biochem.* 181, 545-553.

Utsumi et al. (1988): Structural characterization of fibroblast human interferon-β1. *J. Interferon Res.* 8, 375-384.

Allen, G (1981): Laboratory techniques in biochemistry and molecular biology. *Sequencing of Proteins and Peptides*. Elsevier Verlag.

Castagnota et al. (1988). HPLC in protein sequence determinations. *J. Chromatography* 440, 213-251.

In the peptides designated as (ox), the amino acid methionine is present in the form of methionine sulfoxide. The quantification is based on the determination of the amount of the peak area of the oxidized peptide relative to the total area of the intact peptide and the oxidized peptide. The amounts of oxidized methionines are very low in fresh preparations of interferon-β. During storage, this amount increases to varying extents, depending on the storage conditions (buffer, pH, temperature, etc.). This change is not desirable, because it can contribute to the instability of the interferon-β molecule and it can have a significant influence on the *in vivo* properties.

The amount of oxidized peptides AP4(ox), AP6 (ox), AP8(ox) and AP10(ox) is thus an important criterion for evaluating the chemical integrity of the interferon-β molecule in a liquid formulation.

2. Carbohydrate determination

In the first step, the oligosaccharides were separated from the polypeptide and desalinated.

Approx. 0.7 mL of the eluate from anti-β chromatography was dialyzed against 500 mL dialysis buffer (0.05 mol/L sodium phosphate, 0.10 mol/L NaCl, pH 7.25) for 16 to 20 hours at room temperature while stirring lightly in a dialysis tube (6 mm diameter, Sigma No. D-9277). Then the tube was cut open at one end, and the contents were stripped into an Eppendorf reaction vessel. After dialysis, the volume of the specimen was 1 mL.

To the dialyzed specimen were added 20 µL Tween 20 (10%) and 15 µL N-glycosidase F solution (Boehringer Mannheim) by pipette. This mixture was incubated by for hours at 37 °C. After conclusion of the incubation, the mixture was centrifuged for 10 minutes at 10,000 rpm, filtered through a 0.45 µm

filter and then chromatographed and fractionated over a desalination column (HR 10/10 Pharmacia No. 17-0591-01) with an isocratic gradient (eluent A: distilled water) at a flow rate of 1.0 mL/min. The free oligosaccharides were detected at 206 nm.

In the second step, the oligosaccharides thus released were separated on an ion exchanger and differentiated according to the number of sialic acid groups.

The oligosaccharides contained in the eluate of the desalination column (approx. 2 mL) were bound to an anion exchanger (Mono Q HR 5/5, Pharmacia No. 17-0546-01). The asialo forms are found in the eluate. Monosialo, disialo and trisialo forms eluted with the help of a shallow NaCl gradient are found definitely separated one after the other in the order given here.

Eluent A: Milli-Q water

Eluent B: 0.10 mol/L NaCl

Gradient:

0 min	100 % A	0 % B
5 min	100 % A	0 % B
25 min	33 % A	67 % B
26 min	100 % A	0 % B

Flow rate: 0.75 mL/min

Running time: 26 min (with regeneration 36 min)

Detection: UV 206 nm

Detection of the individual oligosaccharide fractions was performed by means of a UV detector at 206 nm. The quantitative calculation was performed by integration of the areas of the individual peaks.

The monosialo, disialo and trisialo oligosaccharides were then passed through a desalination column as described above.

Then in the third step, the charged oligosacchardes were converted to neutral oligosaccharides by hydrolytic cleavage of the terminal sialic acid groups under acidic pH conditions.

To do so, approx. 15 µL of each oligosaccharide fraction and 15 µL Milli-Q water were placed in a micro test tube, and 30 µL 10 mmol/L H_2SO_4 was added. Then the mixture was heated to 80 °C for 90 minutes.

Next, the test tube was centrifuged at 5000 rpm for one minute, and the batch was pipetted into a mini-vial. The carbohydrates, which were then neutral, were bound to weak anions and to an anion exchange column (CarboPac PA1 (4 × 250 mm) P/N 35391, Dionex) at an alkaline pH. Elution was performed with a gradient as follows:

Eluent A: NaOH 0.15 mol/L

Eluent B: NaOH 0.15 mol/L sodium acetate 0.10 mol/L

Eluent C: NaOH 0.15 mol/L sodium acetate 0.75 mol/L

Gradient:

0 min	95 % A	5 % B	0 % C
2.0 min	95 % A	5 % B	0 % C
3.0 min	85% A	15 % B	0 % C
4.0 min	85 % A	15 % B	0 % C
28.0 min	37 % A	63 % B	0 % C
28.1 min	90 % A	0 % B	10 % C
45.0 min	20 % A	0 % B	80 % C
45.1 min	95 % A	5 % B	0 % C
50.0 min	95 % A	5 % B	0 % C

Flow rate: 1.0 mL/min

Running time: 50 min

Detection: PAD

PAD (pulsed amperometric detection) was used to determine the oligosaccharides. In this method, the oligosaccharide molecule is oxidized electrochemically and the resulting electric current is measured. PAD is an extremely sensitive method which permits detection in the ng range with no problem. The starting signal on the detector (in mV) is directly proportional to the carbohydrate content. Quantification is based on integration of the peak areas.

The specimens were stored temporarily at –20 °C between deglycosylation and analysis.

Literature

Townsend (1988): High performance anion exchange chromatography of oligosaccharides. *Analytical Biochemistry* 174, 459-470.

Results

1. Lys-C mapping

Lys-C mapping of batches 11 through 16 did not reveal any difference in comparison with the initial value with regard to the retention time or qualification [sic; quantification] of the peptides.

Determination of the methionine sulfoxide content during liquid storage yielded the results shown in Table 7 (storage for three months) and Table 8 (storage for six months).

Table 7

Identification	Amount of AP4ox	Amount of AP6ox	Amount of AP8ox	Amount of AP10ox
t_0 value	< 5 %	7.6 %	LOD	LOD
Formulation 11	7.3 %	10.5 %	LOD	LOD
Formulation 12	< 5 %	11.6 %	LOD	LOD
Formulation 13	< 5 %	7.3 %	LOD	LOD
Formulation 14	< 5 %	9.4 %	LOD	LOD
Formulation 15	< 5 %	8.6 %	LOD	LOD
Formulation 16	< 5 %	10.8 %	LOD	LOD

(LOD = not detectable)

Table 8

Identification	Amount of AP4ox	Amount of AP6ox	Amount of AP8ox	Amount of AP10ox
t_0 value	< 5 %	7.6 %	LOD	LOD
Formulation 10	7.6 %	8.9 %	LOD	LOD
Formulation 11	7.7 %	9.6 %	LOD	LOD
Formulation 12	12.0 %	13.7 %	LOD	LOD
Formulation 13	7.4 %	8.7 %	LOD	LOD
Formulation 14	13.7 %	15.7 %	LOD	LOD
Formulation 15	7.4 %	7.9 %	LOD	LOD
Formulation 16	18.0 %	17.0 %	LOD	LOD

It can be seen from Table 7 that after three months of storage, batches 13 and 15, which contain methionine, have a lower methionine sulfoxide content in comparison with the methionine-free batches. After six months of storage, the influence of the added methionine in batches 11, 13 and 15 is more apparent. Only a very slight increase in methionine sulfoxide content can be detected there. The methionine sulfoxide content increases somewhat more in the batches that do not contain any methionine initially, but it remains less than 10 % in the sum of all the oxidized methionine contents relative to the total methionine content.

2. Carbohydrate determination

Tables 9a, 9b, 10a, 10b, 11a and 11b show the results of the carbohydrate determinations after three months of storage and after six months of storage.

On its amino acid chain, interferon-β-1a has a carbohydrate structure which is composed of a defined sequence of monosaccharides. Depending on the type of branching, these are called biantennary structures (having two arms), triantennary structures (three arms) and tetraantennary structures (four arms).

The carbohydrate structure is composed of the monosaccharides mannose, fucose, N-acetylglucosamine, galactose and sialic acid.

Sialic acid is in a special position in several regards:

- It is the only monosaccharide having a charged group (carboxyl group).
- It always occurs in the terminal position and the carbohydrate chain.
- It is split off much more easily by enzymes or by hydrolysis than the other monosaccharides.

- Although the structure of the neutral carbohydrate chain is very constant, there are great variations in the amount of sialic acid, depending on the cell structure and the interferon purification method, among other things.

Literature:

Kagawa et al., *J. Biol. Chem.* 263 (1988), 17508-17515; European Patent EP-A-0 529 300.

The sialo status (percentage amount of individual sialo structures) was investigated after three months of storage (Table 9a) and after six months of storage (Table 9b). A carbohydrate structure which does not have any sialic acid in terminal position is known as an asialo. A carbohydrate structure having one sialic acid group in terminal position is a monosialo. A carbohydrate structure having two sialic acid groups in terminal position is a disialo. A carbohydrate structure having three sialic acid groups in terminal position is a trisialo.

In addition, the antennarity (percentage amount of individual types of branching) was determined after three months of storage (Table 10a) and after six months of storage (Table 10b). A carbohydrate structure having branching and thus having two terminal galactoses is referred to as a biantennary structure. It may be occupied terminally with anything between two sialic acid groups and none. A carbohydrate structure having two branchings and thus terminal galactoses is referred to as a triantennary structure. It may be occupied terminally by zero to three sialic acids.

In addition, the degree of sialylation (percentage occupancy of terminal galactose groups with sialic acid) was also determined after three months of storage (Table 11a) and after six months of storage (Table 11b).

The results show that a slight but reproducible disialylation occurs in storage at a pH of 5. Storage at a pH of 7 does not affect the degree of sialylation.

The afuco component indicated in batches 15 and 16 presumably originates from foreign proteins from the added serum albumin, which was not separated completely by anti-β chromatography.

With regard to the antennarity, there is no measurable influence due to the liquid storage.

Table 9a

Identification	Asialo	Monosialo	Disialo	Trisialo
t_0 value	< 3	13.4	73.4	12.1
Formulation 11	< 3	14.0	74.1	11.9
Formulation 12	< 3	12.5	74.9	11.6
Formulation 13	< 3	16.5	70.4	12.0
Formulation 14	< 3	16.6	71.1	11.1
Formulation 15	< 3	15.8	70.0	13.0
Formulation 16	< 3	15.1	72.0	11.9

Table 9b

Identification	Asialo	Monosialo	Disialo	Trisialo
t_0 value	< 3	13.4	73.4	12.1
Formulation 10	< 3	13.9	70.2	15.3
Formulation 11	< 3	14.5	73.9	11.0
Formulation 12	< 3	14.0	72.4	13.5
Formulation 13	< 3	18.6	68.9	11.7
Formulation 14	< 3	19.0	69.4	10.7
Formulation 15	< 3	17.0	71.0	11.3
Formulation 16	< 3	16.1	71.5	12.4

Table 10a

Identification	Biantennary	Triantennary 1 > 6	Triantennary + 1 repeat
t_0 value	74.4	18.1	3.7
Formulation 11	72.9	18.7	3.7
Formulation 12	76.9	17.0	2.7
Formulation 13	74.7	18.0	3.1
Formulation 14	75.9	17.3	2.9
Formulation 15	75.2 (incl. 5 % afuco)	18.0	3.3
Formulation 16	75.9 (incl. 5 % afuco)	17.8	3.0

Table 10b

Identification	Biantennary	Triantennary 1 > 6	Triantennary + 1 repeat
t_0 value	74.4	18.1	3.7
Formulation 10	71.4	19.3	4.0
Formulation 11	73.0	18.7	3.3
Formulation 12	72.3	19.7	3.4
Formulation 13	72.4	19.2	3.4
Formulation 14	74.2	18.7	3.2
Formulation 15	73.0	18.7	2.8
Formulation 16	74.3 (incl. 4 % afuco)	19.7	3.2

Table 11a

Identification	Degree of sialylation
t_0 value	88.3
Formulation 11	87.0
Formulation 12	88.2
Formulation 13	85.8
Formulation 14	85.8
Formulation 15	86.6
Formulation 16	86.9

Table 11b

Identification	Degree of sialylation

t_0 value	88.3
Formulation 10	87.5
Formulation 11	86.6
Formulation 12	87.7
Formulation 13	84.1
Formulation 14	84.3
Formulation 15	85.7
Formulation 16	86.5

Claims

1. A liquid formulation which contains human interferon-β as an active ingredient in a concentration of up to 25 MU/mL and contains a buffer for adjusting the pH value at 5 to 8, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage at 25 °C for three months.

2. The liquid formulation containing human interferon-β as the active ingredient and a buffer for adjusting the pH value at 6 to 7.2, is free of human serum albumin and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage at 25 °C for three months.

3. The liquid formulation which contains human interferon-β as the active ingredient, a buffer for adjusting the pH value at 5 to 8 and one or more amino acids, and has a long term stability of the biological activity (*in vitro*) of at least 80 % of the initial activity after storage for three months for 25 °C.

4. The formulation according to Claim 1, characterized in that it contains a glycosylated interferon-β.

5. The formulation according to Claim 2, characterized in that the interferon-β originates from CHO cells.

6. The formulation according to one of Claims 1 through 5, characterized in that it contains the buffer in a concentration of 10 mmol/L to 1 mol/L.

7. The formulation according to one of Claims 1 through 6, characterized in that it contains a buffer selected from the group consisting of phosphate buffers, citrate buffers and acetate buffers and mixtures thereof.

8. The formulation according to Claim 7, characterized in that it contains a phosphate/citrate buffer.

9. The formulation according to one of Claims 1 and 3 through 8, characterized in that it has a pH between 6 and 7.2.

10. The formulation according to Claim 3, characterized in that it is free of human serum albumin.

11. The formulation according to one of Claims 1 through 10, characterized in that it is free of human or animal polypeptides apart from the active ingredient.

12. The formulation according to one of Claims 1 through 11, characterized in that it is free of surface-active compounds.

13. The formulation according to one of Claims 1 through 12, characterized in that it has chemical integrity after storage for six months at 25 °C.

14. The formulation according to one of Claims 1 through 13, characterized in that it has physical integrity after storage for six months at 25 °C.

15. The formulation according to one of Claims 1, 2 and 4 through 14, characterized in that it also contains one or more amino acids.

16. The formulation according to Claim 3 or 15, characterized in that it contains methionine.

17. The formulation according to Claim 16, characterized in that the methionine is present in a concentration of 0.1 to 4 mmol/L.

18. The formulation according to one of Claims 1 through 17, characterized in that it also contains additives for adjusting the tonicity.

19. The formulation according to one of Claims 1 through 18, characterized in that it also contains thickeners to increase the viscosity.

20. The formulation according to one of Claims 1 through 19, characterized in that it also contains physiologically acceptable preservatives.

21. A pharmaceutical preparation, characterized in that it contains a liquid formulation according to one of Claims 1 through 20.

22. The pharmaceutical preparation according to Claim 21 for oral, parenteral or ophthalmologic application.

23. The pharmaceutical preparation according to Claim 21 or 22 with single doses of 1 to 25 MU.

24. A method of producing a pharmaceutical preparation according to one of Claims 21 through 23, characterized in that a formulation according to one of Claims 1 through 20 and optionally other pharmaceutically necessary additives is prepared and converted to a suitable form of administration.

25. The method of improving the stability of a liquid formulation containing human interferon-β as the active ingredient and a buffer for adjusting the pH value at 5 to 8, characterized in that a formulation which contains one or more amino acids and/or does not contain any human serum albumin is used.

26. The method according to Claim 25, characterized in that the improvement in stability includes an improvement in the long-term stability of the biological activity (*in vitro*), the chemical integrity and/or the physical integrity.

Sequence Protocol

(1) General information:

(i) Applicant

(A) Name: Dr. Rentschler Biotechnologie GmbH

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(C) City: Laupheim

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(F) Zip code: D-88471

(ii) Name of the invention: Liquid interferon-β formulations

(iii) Number of sequences: 14

(iv) Computer readable version:

(A) Data medium: floppy disk

(B) Computer: IBM PC compatible

(C) Operating system: PC-DOS/MS-DOS

(D) Software: PatentIn Release #1.0 version #1.30 (EPA)

(2) Information on sequence ID No.: 1

(i) Sequence characteristics:

(A) Length: 7 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 109-115

(xi) Sequence description: Sequence ID No. 1

Glu Asp Phe Thr Arg Gly Lys

1 5

(2) Information on sequence ID No.: 2:

(i) Sequence characteristics:

(A) Length: 6 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 100-109

(xi) Sequence description: Sequence ID No. 2

Thr Val Leu Glu Glu Lys

1 5

(2) Information on sequence ID No.: 3

(i) Sequence characteristics:

(A) Length: 7 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 46-52

(xi) Sequence description: Sequence ID No. 3

Gln Leu Gln Gln Phe Gln Lys

1 5

(2) Information on sequence ID No.: 4

(i) Sequence characteristics:

(A) Length: 8 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 116-123

(ix) Feature:

(A) Name/key: modified site

(B) Position: 2

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 4

Leu Xaa Ser Ser Leu His Leu Lyc

1 5

(2) Information on sequence ID No.: 5

(i) Sequence characteristics:

(A) Length: 8 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 116-123

(xi) Sequence description: Sequence ID No. 5

Leu Met Ser Ser Leu His Leu Lys

1 5

(2) Information on sequence ID No.: 6

(i) Sequence characteristics:

(A) Length: 12 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 34-45

(ix) Feature:

(A) Name/key: modified site

(B) Position: 3

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 6

Asp Arg Xaa Asn Phe Asp Ile Pro Glu Glu Ile Lys

1 5 10

(2) Information on sequence ID No.: 7

(i) Sequence characteristics:

(A) Length: 11 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 124-136

(xi) Sequence description: Sequence ID No. 7

Arg Tyr Tyr Gly Arg Ile Leu His Tyr Leu Lys

1 5 10

(2) Information on sequence ID No.: 8

(i) Sequence characteristics:

(A) Length: 12 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 34-45

(xi) Sequence description: Sequence ID No. 8

Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys

1 5 10

(2) Information on sequence ID No.: 9

(i) Sequence characteristics:

(A) Length: 14 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 20-33

(xi) Sequence description: Sequence ID No. 9

Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cyc Leu Lys

1 5 10

(2) Information on sequence ID No.: 10

(i) Sequence characteristics:

(A) Length: 19 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 1-19

(ix) Feature:

(A) Name/key: modified site

(B) Position: 1

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 10

Xaa Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln

1 5 10 15

Cys Gln Lys

(2) Information on sequence ID No.: 11

(i) Sequence characteristics:

(A) Length: 19 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 1-19

(xi) Sequence description: Sequence ID No. 11

Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln

1 5 10 15

Cys Gln Lys

(2) Information on sequence ID No.: 12

(i) Sequence characteristics:

(A) Length: 30 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule:. peptide

(viii) Position in the genome:

(B) Map position: 137-166

(xi) Sequence description: Sequence ID No. 12

Glu Tyr Ser His Cys Ala Trp Thr Ile Val Arg Val Glu Ile Leu Arg

1 5 10 15

Asn Phe Tyr Phe Ile Asn Arg Leu Thr Gly Tyr Leu Arg Asn

20 25 30

(2) Information on sequence ID No.: 13

(i) Sequence characteristics:

(A) Length: 47 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 53-99

(ix) Feature:

(A) Name/key: modified site

(B) Position: 10

(D) Other information: /product "Xaa = Met (oxidized)"

(xi) Sequence description: Sequence ID No. 13

Glu Asp Ile Als Leu Thr Ile Tyr Glu Xaa Leu Gln Asn Ile Phe Als

1 5 10 15

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glue Thr Ile Val

20 25 3

Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ilc Asn His Leu Lys

35 40 45

(2) Information on sequence ID No.: 14

(i) Sequence characteristics:

(A) Length: 47 amino acids

(B) Type: amino acid

(C) Form of strand: single strand

(D) Topology: linear

(ii) Type of molecule: peptide

(viii) Position in the genome:

(B) Map position: 53-99

(xi) Sequence description: Sequence ID No. 14

Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln Asn Ile Phe Ala

1 5 10 1

Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn Glu Thr Ile Val

20 25 30

Glu Asn Leu Leu Als Asn Val Tyr His Cln Ile Asn His Leu Lys

35 40 45